Dependence of the carbon-isotope contents of breath carbon dioxide, milk, serum and rumen fermentation products on the $\delta^{13}C$ value of food in dairy cows

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Six dairy cows of two breeds were fed during three alternating periods with products from C_3- and C_4-plants to yield different natural ^{13}C enrichments of the diet ($\delta^{13}C$ range: −28·0 to −13·7‰). The resulting changes in the ^{13}C enrichment of breath carbon dioxide, serum and milk of the animals followed the $^{13}C:^{12}C$ of the food, in agreement with the individual biological half-lives of those products, and established isotope discriminations. Breath CO_2 was more enriched in ^{13}C than expected. This could be related to isotope discriminations during rumen fermentation. From these results an isotopic balance model for the breath CO_2 could be established.

Carbon isotope discrimination: C_3- and C_4-plants: Cow

The carbon isotope ratio of body tissues in heterotrophic organisms is determined by diet, except for small shifts due to isotope effects in intermediary metabolism (De Niro & Epstein, 1978; Fry *et al.* 1978; Teeri & Schoeller, 1979; Tieszen *et al.* 1979; Miller *et al.* 1985). The $\delta^{13}C$ value (the carbon isotope ratios are reported in the δ-notation: $\delta^{13}C\ (‰) = \{[(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}] \div (^{13}C:^{12}C)_{standard}\} \times 1000$, where standard is a Pee Dee Belemnite carbonate (PDB) (Craig, 1957)) of diets is derived from the proportion of contents from C_3- or C_4-plants, or both. These present different isotope effects during the primary photosynthetic fixation of carbon dioxide (C_3-plants, e.g. wheat, potatoes, soya bean: $\delta^{13}C = -32$ to $-24‰$; C_4-plants, e.g. maize, sugar cane; $\delta^{13}C = -16$ to $-10‰$; Hatch & Slack, 1970; Schmidt, 1986).

The influences of the $^{13}C:^{12}C$ ratios of foodstuffs on the ^{13}C content of body tissues and excretory products in various species have been studied (Jones *et al.* 1979, 1981; Pelletier *et al.* 1984; Schroeder & Plavnik, 1984; Schroeder & Ben-Ghedalia, 1986), but specific features of ruminant digestion have not been investigated in detail. The objectives of the present study, therefore, were to pursue the changes of the ^{13}C content of milk, serum, breath CO_2 and rumen fermentation products, following systematic variations of the ^{13}C content of the diets. Diets were based on naturally labelled material, and enabled estimation to be made of the contribution of rumen fermentation to the $\delta^{13}C$ values of breath CO_2 in cattle.

MATERIAL AND METHODS

Animals, diets and experimental design

Six gravid, lactating cows of Deutsches Fleckvieh (FV) and Deutsche Schwarzbunte (SB) breeds were housed in individual stalls on sawdust. All animals were in the 5th and 6th months of their first lactation. In consequence they were expected to be in positive energy

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Table 1. *$\delta^{13}C$ values ($‰_{PDB}$)* of the diets, daily net energy (NEL) and crude protein (nitrogen × 6·25) intakes of dairy cows and the contribution to energy and protein intakes from diets (A–E) adjusted to specific milk yields*

($\delta^{13}C$ values of diets were calculated from that of the contribution of individual components, see Table 2)

Milk yield (kg/d)	Diet†	$\delta^{13}C$ ($‰_{PDB}$)	NEL (MJ)	Crude protein (g)	Contribution (%) of maize to total diet: NEL	Contribution (%) of maize to total diet: Crude protein
20	A	−28·3	98·8	2265	0	0
	B	−25·9	102·1	2242	10·8	4·9
	C	−24·1	99·3	2194	22·2	10·0
	D	−15·8	103·1	2296	85·0	88·9
	E	−13·7	103·1	2211	95·7	96·8
25	A	−28·0	114·6	2715	0	0
	B	−26·0	115·8	2579	9·5	4·3
	C	−24·7	115·2	2608	19·1	8·4
	D	−15·9	117·5	2698	84·6	89·0
	E	−13·7	116·6	2630	95·5	96·8
30	A	−27·7	130·4	3075	0	0
	B	−26·0	133·4	2990	9·9	4·4
	C	−24·5	133·4	2990	19·8	8·8
	D	−16·2	126·8	3045	83·8	88·8
	E	−13·8	126·8	3093	95·3	96·8

PDB, Pee Dee Belemnite carbonate; A, C_3-plant diet: B, C and D, C_3- and C_4-plant diets; E, C_4-plant diet.

* $\frac{(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}}{(^{13}C:^{12}C)_{standard}} \times 1000$, where standard is PDB (Craig, 1957).

† For details of composition, see Table 2.

and nitrogen balance. Before the experimental periods the cows were supplied with a mixed C_3- and C_4-diet, where approximately half the energy was supplied by maize silage. The rest of the energy was given as hay, grass silage and concentrates, supplemented with minerals, according to requirements. The foodstuffs were obtained from farms near Freising, Bavaria, or purchased at local markets. The nutrient supply in the experimental diets was adjusted to the individual milk yield (Table 1). Water was available *ad lib.* To every diet an appropriate mineral mixture was added. The animals were randomly divided into two groups, and a change-over design was adopted: two main diets A and E were used; transitions between these diets were achieved by use of intermediate diets (B, C and D) for 1 week to avoid digestion problems (Table 2). During the first experimental period, group 1 (2 FV, 1 SB, animal nos. 1–3) received a C_4-plant diet (ration E, $\delta^{13}C$ −13·7‰), in the second period a C_3-plant diet (ration A, $\delta^{13}C$ −28·0‰) and in the third period again a C_4-plant diet (ration E). Group 2 (1 FV, 2 SB, animal nos. 4–6) were treated in the converse manner. Each main diet was given for 2 weeks, following a 1-week change-over.

The composition of the diets and $\delta^{13}C$ values (‰) of their components are presented in Table 2.

Statistics

Results of isotope-ratio analysis are given as means with standard deviations. As the size of the groups was quite small, an analysis of variance seemed inappropriate.

Table 2. *Individual constituents of the whole diet (g/kg dry matter) for a dairy cow with 25 kg milk yield/d and their $\delta^{13}C$ values ($‰_{PDB}$)**

Diet	Maize silage	Maize gluten	Ground maize	Grass silage	Hay	Rolled oats	Sugar-beet pulp	Soya-bean meal
A	—	—	—	344	362	162	65	64
B	103	—	—	156	263	167	225	63
C	202	—	—	153	307	160	85	86
D	575	128	93	—	203	—	—	—
E	654	133	152	—	62	—	—	—
$\delta^{13}C$ ($‰_{PDB}$)	−12·6	−13·9	−12·6	−28·2	−28·3	−27·9	−26·7	−25·6

PDB, Pee Dee Belemnite carbonate; A, C_3-plant diet; B, C and D, C_3- and C_4-plant diets; E, C_4-plant diet.

* $\frac{(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}}{(^{13}C:^{12}C)_{standard}} \times 1000$, where standard is PDB (Craig, 1957).

Sampling and procedures for isotopic analysis

Samples of each component of the ration were taken at the beginning and the end of every period and lyophilized and stored, if not immediately combusted. Sampling of milk was done daily, breath CO_2 samples were taken at weekly intervals and, in transitions between main diets, daily as indicated in Figs. 2 (p. 191) and 3 (p. 192). A pooled sample of milk was taken from the milk churn and frozen; for combustion, 100 μl were dried at 60°. Blood samples (30 ml), obtained from an udder vein, were taken at the end of the periods on the main diets. They were stored at 6° for 2 h, then centrifugated at 1200 ***g*** for 15 min. The serum was removed and lyophilized. All organic samples were combusted with oxygen by established procedures (Winkler & Schmidt, 1980) to CO_2 in an elemental analyser, which consists of an oven at 800°, filled with cupric oxide and cerium oxide and a second oven at 600°, filled with copper for the reduction of nitrogen oxides. After drying over phosphorus pentoxide, CO_2 was separated cryogenically in a vacuum line and analysed by isotope ratio mass spectrometry (MM 903; VG Isogas Ltd, Middlewich, UK) against laboratory CO_2 standards calibrated against PDB (Craig, 1957).

Breath CO_2 was collected by means of a face mask, connected by a two-way valve to a gas-tight bag (Linde AG, Munich, FRG). After a 5 min equilibration period, breath was pooled for 2 min in the bag. By means of an adapter, the gas was transfered to 15-ml Vacutainers® (Becton-Dickinson GmbH, Heidelberg, FRG) (Schoeller & Klein, 1978). The breath samples were dried over P_2O_5, and the CO_2 was purified by cryogenic techniques in a vacuum line, then expanded into the inlet system of the mass spectrometer for $^{13}C:^{12}C$ ratio measurement.

Rumen-simulation experiments

For the simulation of rumen fermentation, 50 ml roughly strained rumen fluid from rumen-fistulated wethers, given 400 g hay and 600 g concentrates/d, were incubated for 3–8 h with 2 g milled substrates and 50 ml 0·5 M-phosphate buffer (pH 6·5) in a 250 ml three-necked flask at 39°.

The mixture was stirred while helium was bubbled through, establishing anaerobic conditions. The gases produced passed through a trapping and combustion system (Fig. 1), where moisture was condensed at −100° (liquid N_2 in isopentane), and CO_2 was isolated in a removable trap at −160° (liquid N_2 in isopentane). The residual gas was washed with

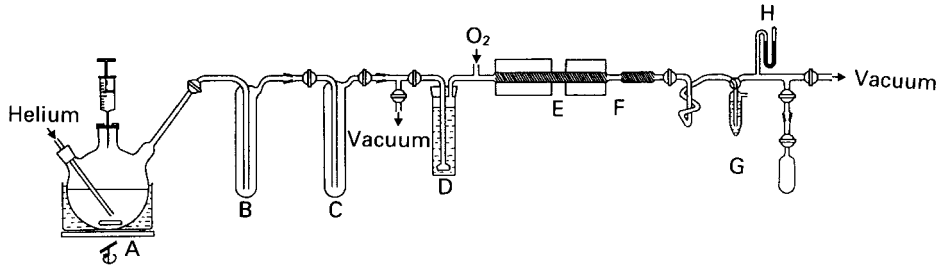

Fig. 1. Apparatus for the in vitro fermentation of substrates with rumen fluid and the collection of the generated carbon dioxide and methane. A, incubation flask; B, moisture trap, −100°; C, removable trap for CO_2 collection, −160°; D, wash flask, barium hydroxide; E, combustion unit (cupric oxide, 800°); F, phosphorus pentoxide–pumice for water absorption; G, system for trapping and recondensation (−196°) of combustion CO_2; H, mercury manometer.

barium hydroxide and passed with O_2 through the elemental analyser mentioned previously (Winkler & Schmidt, 1980), where methane was combusted to CO_2, which was collected by cryogenic means. The isotope ratio of the CO_2 samples was analysed as mentioned previously (SIRA 24; VG Isogas Ltd). The CO_2:CH_4 ratio in the fermentation gases was determined by means of gas–liquid chromatography (Dani 3800; Dani S.p.A., Monza, Italy; columns: Porapack Q and Porapack R in sequence, 2 m each; oven temperature 50°, isotherm; carrier gas He, 15 ml/min; thermal conductivity detector).

Volatile fatty acids (VFA) were isolated from the incubation medium before and after fermentation by steam distillation; the distillate was neutralized and the sodium salts were dried and combusted to CO_2, which was analysed for ^{13}C content as described previously.

RESULTS

$\delta^{13}C$ values of milk and serum

The $\delta^{13}C$ value of whole milk directly reflected that of the diet (Fig. 2). Particularly at the end of the pure C_4-feeding period (ration E), a coincidence of the $\delta^{13}C$ values of milk and foodstuff was attained, while in the course of giving the C_3-ration A, the milk was relatively enriched in ^{13}C by 1·5‰ (Table 3). The maximum and minimum $\delta^{13}C$ values obtained in milk were −13·1 and −27·4‰ respectively.

Total serum C, starting at a mean $\delta^{13}C$ value of −17·3‰ at the beginning of the experimental period, varied between −16 and −22‰, following the changing diets (Table 3).

During the pre-experimental period, where an equilibration of several months to a diet of −23‰ occurred, milk and serum showed a systematic ^{13}C enrichment relative to diet of 1·3 and 5·7‰, respectively (Fig. 2, Table 3). As the diet was given *ad lib.* during this period, the variation of observed δ values was significantly higher than with the main diets.

All results were independent of the breed, and individual variations observed could be tentatively related to the type of food consumed by the animals, or to different fat content of the milk.

$\delta^{13}C$ values of breath CO_2, and rumen CO_2, CH_4 and VFA

Diet change induced corresponding alterations in the $\delta^{13}C$ values of breath CO_2 (Fig. 3, Table 3). However, in contrast to the known relative ^{13}C depletion found with breath CO_2 of non-ruminants (De Niro & Epstein, 1978; Metzler *et al.* 1983), the ^{13}C content in breath was equal to that of ration E (C_4-plants) or slightly enriched, but was markedly enriched (by 4–5‰) when C_3-plants (ration A) were consumed.

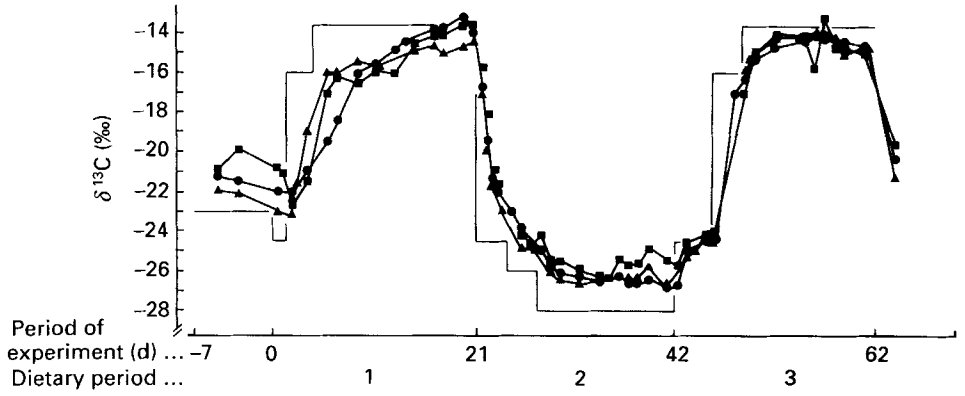

Fig. 2. $\delta^{13}C$ values ($‰_{PDB}$) of the diet (——) and the milk of three individual cows (group 1: animal nos. 1 (▲), 2 (●), 3 (■)). $\delta^{13}C$ (‰) = $\{[(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}] \div (^{13}C:^{12}C)_{standard}\} \times 1000$, where standard (PDB) is Pee Dee Belemnite carbonate (Craig, 1957). Period 1, C_4-plant diet; period 2, C_3-plant diet; period 3, C_4-plant diet.

Table 3. *Mean $\delta^{13}C$* values ($‰_{PDB}$) of total carbon of milk, serum and breath of dairy cows achieved with the given $\delta^{13}C$ values ($‰_{PDB}$) of the main diets A and E*†

(Means and standard deviations)

		Milk (*n* 3)		Serum (*n* 2)		Breath (*n* 3)	
	Diet	Mean	SD	Mean	SD	Mean	SD
Pre-experimental period	−23·0	−21·7	0·72	−17·3	2·10	−19·3	1·90
Group 1							
E	−13·7	−13·9	0·40	−15·9	0·36	−11·9	0·15
A	−28·0	−26·2	0·62	−20·5	0·37	−24·0	0·59
E	−13·7	−14·6	0·17	−17·0	0·40	−13·9	0·51
Group 2							
A	−28·0	−27·3	0·40	−21·8	0·33	−22·6	0·22
E	−13·7	−14·0	0·36	−17·4	0·40	−13·4	0·25
A	−28·0	−26·5	0·16	−19·9	0·24	−24·9	0·79

PDB, Pee Dee Belemnite carbonate; A, C_3-plant diet; E, C_4-plant diet: group 1, cow nos. 1–3; group 2, cow nos. 4–6.

* $\dfrac{(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}}{(^{13}:^{12}C)_{standard}} \times 1000$, where standard is PDB (Craig, 1957).

† For details of composition, see Table 2.

Similarly the $\delta^{13}C$ values of breath CO_2 during the pre-experimental period (equilibration to a diet of −23‰) exceeded that of the ^{13}C content in the diet by up to 4‰. Therefore, the consequences of fractionation phenomena of the rumen fermentation were examined to provide an estimation of the isotopic balance of the C metabolism in cows.

Generally for CH_4-producing ecological systems (sediments of lakes, soils and marine aquatic systems), considerable fractionations of C isotopes have been observed during the anaerobic breakdown of organic matter (Rosenfeld & Silverman, 1959; Games *et al.* 1978; Bryant, 1979; Schoell, 1980). A corresponding phenomenon may be expected with rumen digestion and, indeed, Rust (1981) has observed high ^{13}C depletions in CH_4 from air around

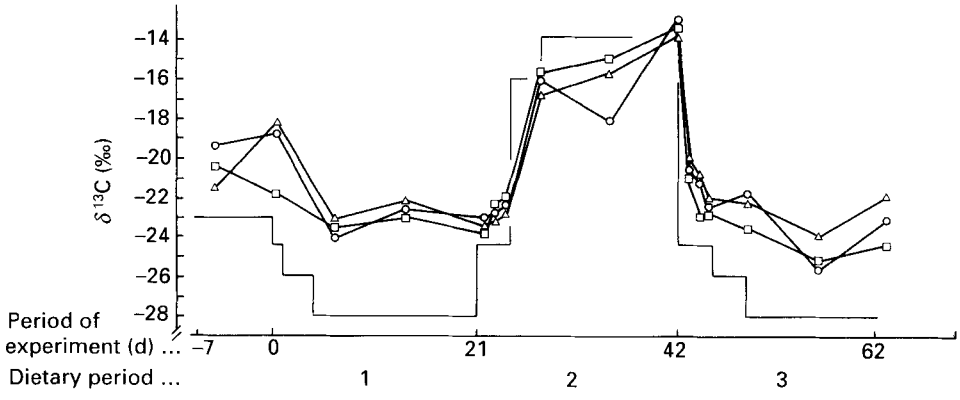

Fig. 3. $\delta^{13}C$ values ($‰_{PDB}$) of the diet (——) and the breath carbon dioxide of three individual cows (group 2: animal nos. 4 (△), 5 (○), 6 (□)). $\delta^{13}C$ (‰) = $\{[(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}] \div (^{13}C:^{12}C)_{standard}\} \times 1000$, where standard (PDB) is Pee Dee Belemnite carbonate (Craig, 1957). Period 1, C_3-plant diet; period 2, C_4-plant diet; period 3, C_3-plant diet.

Table 4. *$\delta^{13}C$ values* ($‰_{PDB}$) of the fermentation products carbon dioxide, methane and volatile fatty acids (VFA) of dairy cows after 3–8 h† in vitro incubation of C_3- and C_4-plant material (2 g) with rumen fluid (50 ml) of C_3-plant origin‡*

Substrate	$\delta^{13}C$ ($‰_{PDB}$)	Period of incubation (h)	$\delta^{13}C$ values ($‰_{PDB}$)		
			CO_2	CH_4	VFA§
Beet sucrose	−25·9	3	−8·0	−62·3	−27·4
Dried sugar-beet pulp:					
1	−26·9	5	−9·5	−75·6	−26·6
2	−26·9	8	−8·7	−70·6	−27·2
Hay	−28·5	6	−8·1	−72·1	−28·0
C_3:					
Mean	−27·1		−8·6	−70·2	−27·3
SD	1·1		0·7	5·6	0·6
Maize glucose	−10·7	5	+1·3	−56·0	−20·1
Ground maize:					
1	−12·2	6	−0·6	−55·5	−19·7
2	−12·2	6	−5·4	−52·9	−18·3
Maize cob husks	−12·9	7	+0·5	−60·5	−21·1
C_4:					
Mean	−12·0		−1·1	−56·2	−19·8
SD	0·9		3·0	3·2	1·2

PDB, Pee Dee Belemnite carbonate.

* $\frac{(^{13}C:^{12}C)_{sample} - (^{13}C:^{12}C)_{standard}}{(^{13}C:^{12}C)_{standard}} \times 1000$, where standard is PDB (Craig, 1957).

† Fermentation periods for the easily digestible carbohydrates (beet sucrose, maize glucose) were shortened because of the rapid decrease of pH and the consequences of this on the microbial ecosystem.

‡ $\delta^{13}C$ value of the diet of the donor animals −27·6‰.

§ $\delta^{13}C$ value of VFA isolated from the original rumen fluid −26·0‰.

cattle stalls relative to the digested food. As the variables (e.g. fat depot flux, gas production) required for the establishment of an isotope balance are rather complicated in vivo, we have separated rumen fermentation from tissue metabolism. This was based on fermentations in vitro of various foodstuffs followed by isotopic analysis of the generated CO_2, CH_4 and VFA. A major C-isotope discrimination was observed with the fermentation process; the $\delta^{13}C$ values of the liberated CH_4 gas were on average 43·1 and 44·2‰ more negative, while those of the CO_2 were 18·5 and 10·9‰ more positive than those of the respective C_3- and C_4-substrates (Table 4). For the VFA, the C-isotope ratios tended towards those of the digested C_3-substrates (Table 4), but was rather different when C_4-substrates were used. This may be related to the use of rumen fluid from wethers fed on C_3-plants and may represent a situation similar to the influence of endogenous pools in the course of the in vivo experiments.

DISCUSSION

In an attempt to establish an isotopic balance for CO_2 in lactating ruminants, one has to take into consideration a pronounced difference compared with simple-stomached animals. In general, the latter produce slightly depleted breath CO_2 (by about 2‰) and faeces relative to the diet, whereas the body tissues and secretions show a corresponding ^{13}C enrichment (De Niro & Epstein, 1978; Jones *et al.* 1981; Metzler *et al.* 1983; Schroeder & Ben-Ghedalia, 1986). This ^{13}C enrichment in body tissues, which must be related to isotopic fractionations during metabolic reactions, has also been observed in this investigation in ruminants. During the pre-experimental equilibration period, milk and serum attained a ^{13}C enrichment of 1·3 and 5·7‰ respectively (Fig. 2, Table 3); the ration switch between C_3- and C_4-plant material effected changes in enrichment relative to the pre-experimental period, the size of the change depending on biological half-life. This is in contrast to the results of Boutton *et al.* (1988) who reported, for a similar experimental design, a slight ^{13}C depletion in milk; the discrepancy with our results cannot as yet be explained.

The enriched milk and serum was not balanced by a correspondingly depleted breath CO_2, on the contrary, the latter showed enrichments up to 4‰ during the equilibrated pre-experimental period and during C_3-plant feeding (ration A). For the C_4-plant feeding (ration E), the $\delta^{13}C$ values of breath CO_2 attained only the level of the diet or an excess of 2‰. For this different effect of C_3- and C_4-plant feeding on breath δ values, three possible explanations may be considered. First, there may be an effect of the feeding history and the relatively short equilibration times for one diet. Second, it must be assumed that the C_3- and C_4-plant diets had different contents of fibre, cellulose, starch, etc., leading to different digestive activity in the intestine, e.g. rumen fermentation and its end-products. We will show later in the present paper that this might be an important factor. A third explanation was suggested by Tyrrell *et al.* (1984), who interpreted similar observation to ours as an expression of preferential oxidation of maize fractions in the ruminant. However, their findings show an enrichment of breath CO_2 even after a pure C_3-plant diet, and our own results demonstrate that the enrichment is especially high after C_3-plant feeding without any maize.

As the CO_2 depletion in tissues of cows must be assumed to be equal to that in non-ruminant animals, there has to be a second source of CO_2 responsible for the ^{13}C shift of the breath CO_2 of cows. Our in vitro experiments provide evidence that this must be a considerably enriched CO_2 released in the rumen. The anaerobic degradation of carbohydrates in the rumen produces VFA, CO_2 and reducing equivalents. Some of the CO_2 is used as a hydrogen acceptor for the formation of CH_4 ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$; Bryant, 1979). As this process is accompanied by a large isotope discrimination, 'light' CH_4 is

produced, and the non-reduced CO_2 must be correspondingly enriched. The results of our simulation experiments are in agreement with this assumption (Table 4).

Based on the in vivo and in vitro experiments, estimates of various contributions to breath-CO_2 $\delta^{13}C$ values can be made. The phase-A diet (pure C_3-plant food) and the incubation of C_3 substrates in the rumen fermentation experiments can be considered to correspond to each other. In vitro, the mean $\delta^{13}C$ value of the VFA ($-27{\cdot}3‰$) was nearly identical to that of the substrates ($-27{\cdot}1‰$; Table 4), indicating that a complete equilibration had been established and, therefore, only the isotopic fractionation between the gases has to be taken into account. The fraction of CO_2 reduced to CH_4 (x) can be calculated to be 30% from isotope balance:

$$\delta^{13}C_{substrate} \times 100 = \delta^{13}C_{CH_4} \times x + \delta^{13}C_{CO_2} \times (100 - x). \quad (1)$$

This reduction rate calculated from isotope balance fits with the result of the gas–liquid chromatographic analysis of the fermentation gases (CO_2:CH_4, 71:29), and is similar to values reported in the literature (Schlegel, 1981). Hence the isotope balance appears to be a reliable tool for the estimation of C fluxes.

In the rumen of a cow of 650 kg body-weight and a daily milk yield of 20 kg, about 2500 g (42 mol) acetic acid, 800 g (11 mol) propionic acid and 450 g (5 mol) butyric acid are produced daily (Kirchgessner, 1985). The stoichiometry and H balance of the rumen fermentation processes involved demand the simultaneous production of 16 mol CH_4 and 36 mol CO_2, a ratio similar to the experimental gas production mentioned previously. Approximately 60% of the energy uptake (equal approximately to the C uptake) is needed for milk production, leaving 40% for maintenance (oxidation). In total, 137 g atom C are available as a source of energy for animal metabolism.

The CO_2 from the rumen fermentation experiment showed a $\delta^{13}C$ value of $-8{\cdot}6‰$. In vivo, a second source of CO_2 production originates from the oxidative metabolism of the animal. An isotope balance for these two pools has to take into account that the milk was ^{13}C-enriched by $1{\cdot}8‰$ ($1{\cdot}5$–$2‰$; Fig. 2, Table 3) relative to the diet in feeding period A. This enrichment is valid for the assumed positive energy balance at the stage of lactation of the experimental cows. (The contribution of endogenous C by normal turnover in tissues cannot be taken into account.) Hence, the $\delta^{13}C$ value of the C pool entering oxidation (x) can be calculated as $-30{\cdot}7‰$ (based on mean $\delta^{13}C$ values for the food and the substrates in vitro):

$$137 \text{ g atom C} \times -28{\cdot}0‰_{diet} = 0{\cdot}6 \times 137 \text{ g atom C} \times -26{\cdot}2‰_{lactation} + 0{\cdot}4 \times 137 \text{ g atom C} \times x‰_{oxidation}. \quad (2)$$

From this equation the contribution of the diet to energy expenditure can be calculated. Assuming a similar isotope discrimination in the oxidative metabolism to that in simple-stomached animals, we must expect the substrate C pool for oxidation to CO_2 to be depleted by 2‰ (which will be compensated by enrichment of tissues and the output through faeces and urine). The total amount of this CO_2 produced by the animal's oxidative metabolism is $0{\cdot}4 \times 137$ g atom C = 55 g atom C (equal to 55 mol CO_2). Summarizing the two CO_2 pools, we are now able to calculate the $\delta^{13}C$ value of CO_2 from oxidation in tissues to be $-32{\cdot}6‰$:

$$55 \text{ mol } CO_2 \times x‰_{oxidation} = 91 \text{ mol } CO_2 \times -23{\cdot}1‰_{breath} - 36 \text{ mol } CO_2 \times -8{\cdot}6‰_{rumen}. \quad (3)$$

This value is very close to the expected $-32{\cdot}7‰$ calculated from equation (2) with consideration of isotope fractionation in oxidative metabolism of 2‰. This fits very well

with the proposed conceptions of the C pools in ruminants and the equilibration of the CO_2 produced in these pools.

According to Hoernicke *et al.* (1965), there are two physiological pathways for rumen CO_2 to enter expired air: the first involves diffusion through the rumen wall into the blood and the second inhalation of eructed rumen gases. Therefore, the difference observed in the $\delta^{13}C$ values of the breath CO_2 of cows relative to non-ruminating animals, i.e. a ^{13}C enrichment running up to 4‰ (depending on the diet) is satisfactorily explained by the isotope discrimination during rumen fermentation. In addition, our results demonstrate, generally, that metabolic tracer experiments can be performed with large animals without the complications inherent in the use of radiotracers, by the alternative approach of using inexpensive sources of differentially ^{13}C-labelled compounds derived from C_3- and C_4-plants.

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